

## Association of Virulence with Antimicrobial Resistance among *Klebsiella pneumoniae* Isolated from Hospital Settings in Bangladesh

### Abstract

**Introduction:** Infections caused by multidrug-resistant (MDR) hypervirulent *Klebsiella pneumoniae* are difficult to treat and associated with high mortality rates. Hence, this study was conducted to determine the antibiotic resistance pattern along with the distribution of virulence genes among isolated string test positive and negative strains. **Materials and Methods:** A total of 44 *K. pneumoniae* strains were isolated following standard microbiological methods from 350 different clinical samples from patients admitted to Dhaka Medical College Hospital, Bangladesh. String test was done to detect the hypermucoid phenotype. Antimicrobial resistance (AMR) pattern was determined by dichlorodiphenyltrichloroethane (except colistin and fosfomycin) among all isolates. Polymerase chain reaction was done to detect the hypervirulence genes (*magA*, *rmpA*, *rmpA2*, *iutA*, *iroN*). **Results:** In this study, 21/44 (47.73%) of the isolated *K. pneumoniae* were string test positive and distribution of the virulence genes except *rmpA2* was higher among them. A total of 15/44 (34.09%) of the isolated *K. pneumoniae* were MDR, 10/44 (22.73%) were extensively drug resistant, 1/44 (2.27%) was pan drug resistant, and 14/44 (31.82%) were colistin resistant. Isolated organisms were highly resistant to third-generation cephalosporins and most sensitive to fosfomycin in this study. Although all the string test positive strains showed higher resistance rates than the string test negative ones toward most of the tested antibiotics, only the differences of resistance rates to amoxiclav and tigecycline among the two phenotypes were statistically significant. **Conclusion:** Our findings highlight the importance of surveillance of the AMR pattern of hypervirulent *K. pneumoniae* in clinical samples. Therefore, a response to check the global dissemination of this hypervirulent *K. pneumoniae* with resistance determinants is urgently needed.

**Keywords:** Antimicrobial resistance, Bangladesh, hypervirulent, *Klebsiella pneumoniae*

### Introduction

A novel and distinct variant of the superbug *Klebsiella pneumoniae* called hypervirulent *K. pneumoniae* (hvKP) has emerged which is predicted to become a major threat in the world including Asia and Western countries.<sup>[1]</sup> In comparison to classical *K. pneumoniae*, hvKP strains exhibit enhanced virulence in terms of overproduction of capsular polysaccharides and antiphagocytosis resulting in severe infections such as pyogenic liver abscess, pneumonia, endophthalmitis, osteomyelitis, and necrotizing fasciitis.<sup>[2]</sup> Moreover, the emergence of multi and pandrug-resistant (PDR) strains renders management of hvKP infections extremely challenging due to the acquisition of extended-spectrum  $\beta$ -lactamases (ESBLs), carbapenemases,<sup>[3]</sup> and recently described *mcr* genes. Most

strains causing pyogenic infections show hypermucoviscosity when grown on agar plates and cause the generation of viscous strings exceeding 5 mm between a colony and a loop; referred to as string test.<sup>[4]</sup> These strains are much more resistant than classic *K. pneumoniae* to *in vitro* killing by serum, to phagocytosis by neutrophils and macrophages, cause liver abscess and meningitis in mice; they have thus been referred to as hvKP strains.

The first gene identified as contributing to the hypermucoviscous phenotype in the representative strain NTUH-K2044 was *magA* (mucoviscosity-associated gene A), which also conferred increased virulence in mouse models.<sup>[5]</sup> In addition to *magA*, several virulence factors including *rmpA* and various siderophore genes have been identified that characterize the hypermucoviscous hypervirulent phenotype. The prototypical hypermucoviscous K1 strain NTUH-K2044

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carries two *rmpA* plasmid-borne genes (*prmpA* and *prmpA2*) on the same large plasmid (pK2044).<sup>[3]</sup> Siderophores are also important virulence factors that scavenge scarce ferric iron from the environment. In *K. pneumoniae*, aerobactin (encoded by *iutA*), salmochelin (also known as catechol receptors) (encoded by *iroN*), and yersiniabactin are the representative siderophores.<sup>[6,7]</sup> The convergence of hypervirulence and multidrug resistance (MDR) in *K. pneumoniae*, through both acquisition of resistance genes by hypervirulent strains and the opposite phenomena, poses a consequential public health threat.<sup>[8]</sup>

Positive string test has been reported as a factor of hypervirulence and no study has so far been carried out to observe antimicrobial resistance (AMR) pattern among string test positive *K. pneumoniae* in Bangladesh. Hence, this study has been done to evaluate the association of hypervirulence with multidrug resistance (MDR) in *K. pneumoniae* at a tertiary care medical college hospital in Bangladesh.

## Materials and Methods

A total of 350 samples (wound swab, sputum, endotracheal aspirates, pus, blood, and urine) were collected following standard protocol and processed further as required from patients admitted to Dhaka Medical College Hospital, Dhaka, Bangladesh, during 2018–2019. Written informed consent was obtained from the patients and the study was approved by the institutional ethical review committee.

### Isolation and identification of the organisms from culture

Phenotypic identification of the organisms was done by observing colony morphology, hemolytic criteria, staining characteristics, pigment production (in Blood and MacConkey agar media), and biochemical tests (oxidase test, catalase tests, and other biochemical reactions after inoculation in Triple Sugar Iron, Motility Indole Urea, and Simmon's Citrate agar media). Isolated *K. pneumoniae* were subcultured in Mueller–Hinton agar media and preserved at  $-22^{\circ}\text{C}$  for further use.

### Antimicrobial susceptibility test and determination of minimum inhibitory concentration

Susceptibility to antimicrobial agents of all isolates was done by modified Kirby-Bauer disc diffusion technique using Mueller–Hinton agar plates and zones of inhibition were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>[9]</sup> The US Food and Drug Administration identified interpretive criteria that were used for the interpretation of the zone of inhibition of tigecycline. The antimicrobial discs were used according to the standard antibiotic panel for isolated organisms. Following antibiotic discs were obtained from commercial source (Oxoid Ltd, UK)-Amoxiclav (amoxicillin 20  $\mu\text{g}$  and clavulanic acid 10  $\mu\text{g}$ ), piperacillin-tazobactam (100/10  $\mu\text{g}$ ),

cefepime (30  $\mu\text{g}$ ), ceftazidime (30  $\mu\text{g}$ ), ceftriaxone (30  $\mu\text{g}$ ), cefuroxime (30  $\mu\text{g}$ ), ceftazidime (30  $\mu\text{g}$ ), amikacin (30  $\mu\text{g}$ ), ciprofloxacin (5  $\mu\text{g}$ ), imipenem (10  $\mu\text{g}$ ), tigecycline (15  $\mu\text{g}$ ), and aztreonam (30  $\mu\text{g}$ ). *Escherichia coli* ATCC 25922 was used as control strain to assess the performance of the method. The Agar dilution method was done to determine the minimum inhibitory concentration (MIC) of colistin, tigecycline, imipenem, and fosfomycin.<sup>[9,10]</sup> Double-disc synergy test was done to detect ESBL-producing strains. MDR, extensively drug resistant (XDR), and PDA strains were identified according to the Center for Disease Control and Prevention (CDC) definitions.

### String test

To detect the hypermucoid phenotype, string test was done using sterile platinum wire loop to lift a colony of *K. pneumoniae* grown on blood agar plate. The length of the viscous string was measured by placing a ruler straight behind it.

## Molecular methods

### Polymerase chain reaction and sequencing

DNA extraction of the preserved samples was done following standard extraction protocol (boiling method). This extracted DNA was kept at  $-22^{\circ}\text{C}$  and was further used for polymerase chain reaction (PCR).

Amplification in the thermal cycler was done using specific primers for virulence (*magA*, *rmpA*, *rmpA2*, *iutA*, *iroN*). Amplified products of PCR were detected by ultraviolet transillumination (Gel Doc, Major science, Taiwan) after electrophoresis on 1.5% agarose gel prepared with 1X TAE buffer and stained with ethidium bromide.

### Data processing

All data were compiled and edited meticulously by thorough checking and rechecking. All omissions and inconsistencies were corrected and were removed methodically.

### Data analysis

The results of the study were recorded systematically. Data were analyzed and compared using Z test. All statistical analysis was performed using SPSS Statistics for Windows, version 15 (SPSS Inc., Chicago, Ill., USA).  $P = 0.05$  was taken as a minimal level of statistical significance.

## Results

A total of 350 clinical samples were included in the present study of which, 220 (62.86%) samples yielded culture positivity. The distribution of total isolated and string test positive *K. pneumoniae* in different samples is shown in Table 1. The distribution of different bacterial species among 220 different culture-positive clinical samples is demonstrated in Table 2.

AMR pattern among total isolated ( $n = 44$ ), string positive ( $n = 21$ ), and string negative ( $n = 23$ )

**Table 1: Total and string positive number of *Klebsiella pneumoniae* isolated from different culture-positive samples**

Samples	Number of samples	Culture positive, n (%)	Isolated n (%) of <i>K. pneumoniae</i>	String test positive <i>K. pneumoniae</i>
Wound swab	97	75 (77.32)	15 (20.00)	7 (46.66)
Urine	91	43 (47.25)	8 (18.60)	4 (50.00)
Sputum	42	23 (54.76)	8 (34.78)	3 (37.50)
ETA	22	19 (86.36)	7 (36.84)	3 (42.86)
Pus	52	36 (69.23)	4 (11.11)	3 (75.00)
Blood	46	24 (52.17)	2 (8.33)	1 (50.00)
Total	350	220 (62.86)	44 (20.00)	21 (47.73)

ETA: Endotracheal aspirates; *K. pneumoniae*: *Klebsiella pneumoniae***Table 2: Distribution of organisms isolated from different samples (n=220)**

Organisms	n (%)
<i>E. coli</i>	54 (24.55)
<i>P. aeruginosa</i>	51 (23.18)
<i>K. pneumoniae</i>	44 (20.00)
<i>K. oxytoca</i>	2 (0.90)
<i>A. baumannii</i>	13 (5.90)
<i>E. cloacae</i>	8 (3.64)
<i>E. aerogenes</i>	6 (2.72)
<i>C. freund</i>	2 (0.91)
<i>C. koseri</i>	1 (0.45)
<i>P. mirabilis</i>	5 (2.27)
<i>P. vulgaris</i>	3 (1.36)
Gram-positive bacteria	31 (14.09)
Total	220 (100.00)

*E. coli*: *Escherichia coli*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *K. pneumoniae*: *Klebsiella pneumoniae*; *K. oxytoca*: *Klebsiella oxytoca*; *A. baumannii*: *Acinetobacter baumannii*; *E. cloacae*: *Enterobacter cloacae*; *E. aerogenes*: *Enterobacter aerogenes*; *C. freund*: *Citrobacter freund*; *C. koseri*: *Citrobacter koseri*; *P. mirabilis*: *Proteus mirabilis*; *P. vulgaris*: *Proteus vulgaris*

*K. pneumoniae* is shown in Table 3. Although higher resistance rates toward all the tested antibiotics (except for ceftriaxone, piperacillin-tazobactam, and colistin) were observed among the string test positive strains than that of string test negative isolates, only resistance proportions to amoxiclav and tigecycline were significantly higher among them ( $P < 0.05$ ). Highest sensitivity was seen toward fosfomycin, followed by imipenem and least *in vitro* effective drug was cefuroxime, followed by ceftriaxone in this study. In Table 4, the MIC ranges of imipenem, tigecycline, colistin, and fosfomycin among resistant *K. pneumoniae* have been shown.

Distribution of MDR, XDR, PDR, and ESBL-producing strains of isolated *K. pneumoniae* in different samples is demonstrated in Table 5 along with their relative frequency among the string test positive and string test negative strains in Figure 1. Among the isolated strains, 15 (34.09%) were MDR, 10 (22.73%) were XDR, and 1 (2.27%) was PDR pathogen.

Distribution of virulence genes among isolated *K. pneumoniae* in different samples [Table 6] has been

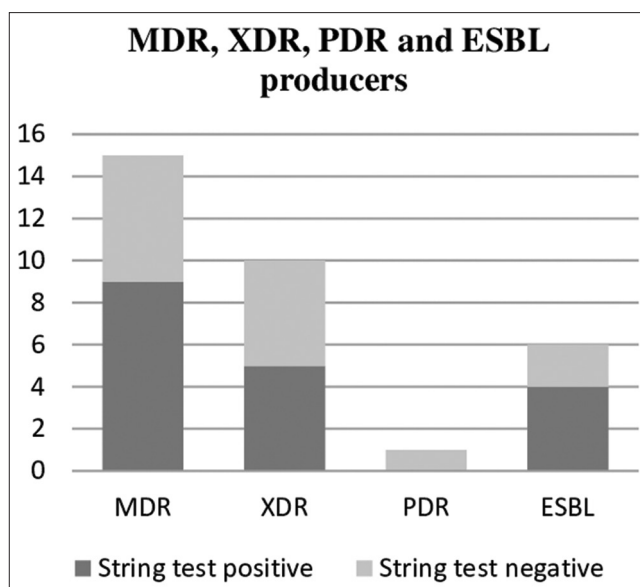


Figure 1: Here, the number of multidrug-resistant and extended-spectrum  $\beta$ -lactamases producing strains was higher among string test positive isolates than the string test negative ones but the difference was only statistically significant for extended-spectrum  $\beta$ -lactamases producers ( $P < 0.00001$ )

demonstrated, and the comparative distribution of these genes among the hypermucooid phenotypes or string positive and string negative strains is shown in Figure 2.

## Discussion

*K. pneumoniae* has been described over the past decades as an opportunistic pathogen associated with the health-care setting, wherein it is capable of causing severe illnesses. It is now considered a reservoir for AMR and virulence genes.<sup>[11]</sup> The acquisition and dissemination of ESBL, carbapenem, and now colistin-resistance encoding genes<sup>[12,13]</sup> make this pathogen an urgent threat to human health. Infections caused by the hypervirulent strains are reported to be associated with higher morbidity and mortality even with antibiotic-sensitive strains.<sup>[14]</sup>

In this study, among 350 different clinical samples, 62.86% yielded culture-positive results. In the present study, among the culture-positive samples, 24.55% were *E. coli* which was the most common pathogen, followed by *Pseudomonas aeruginosa* (23.18%). *K. pneumoniae* accounted to be

**Table 3: Antimicrobial resistance pattern among isolated *Klebsiella pneumoniae* (n=44) with comparison between string positive and string negative isolates**

Antimicrobial drugs	Resistance			P
	Total isolated, n (%)	String test positive, n (%)	String test negative, n (%)	
Amikacin	23 (52.27)	13 (61.90)	10 (43.48)	0.10
Amoxiclav	24 (54.54)	15 (71.43)	9 (39.13)	0.01*
Cefepime	25 (56.82)	13 (61.90)	12 (52.17)	0.25
Ceftazidime	30 (68.18)	16 (76.19)	14 (60.87)	0.14
Ceftriaxone	32 (72.73)	14 (66.66)	18 (78.26)	0.21
Cefoxitin	24 (54.54)	14 (66.66)	10 (43.48)	0.06
Cefuroxime	33 (75.00)	16 (76.19)	17 (73.91)	0.43
Ciprofloxacin	26 (59.09)	15 (71.43)	11 (47.83)	0.06
Piperacillin-tazobactam	18 (40.90)	8 (38.09)	10 (43.48)	0.36
Aztreonam	31 (70.45)	15 (71.43)	16 (69.56)	0.45
Imipenem	9 (20.45)	6 (28.57)	3 (13.04)	0.09
Tigecycline	9 (20.45)	7 (33.33)	2 (8.69)	0.02*
Fosfomycin	6 (13.67)	4 (19.04)	2 (8.69)	0.15
Colistin	14 (31.82)	5 (23.81)	9 (39.13)	0.16

\*statistically significant, (P<0.05)

**Table 4: Minimum inhibitory concentration ranges of imipenem, tigecycline, colistin, and fosfomycin among resistant *Klebsiella pneumoniae* isolates**

Name of antibiotic	Number of resistant isolates	MIC range (µg/ml)
Imipenem	9	16-≥128
Tigecycline	9	8-≥32
Colistin	14	16-≥256
Fosfomycin	6	512-≥4096

CLSI breakpoint of MIC of imipenem for *K.*

*pneumoniae* - Sensitive ≤1 µg/ml, Intermediate 2 µg/ml,

Resistant ≥4 µg/ml; FDA breakpoint of MIC of tigecycline for *K.*

*pneumoniae* - Sensitive ≤2 µg/ml, Resistant ≥8 µg/ml; EUCAST

breakpoint of MIC of colistin for *K. pneumoniae* - Sensitive

≤2 µg/ml, Resistant >2 µg/ml; EUCAST breakpoint of MIC of

fosfomycin for *K. pneumoniae* - Sensitive <32 µg/ml, Resistant

≥32 µg/ml. *K. pneumoniae*: *Klebsiella pneumoniae*; MIC:

Minimum inhibitory concentration; CLSI: Clinical and Laboratory

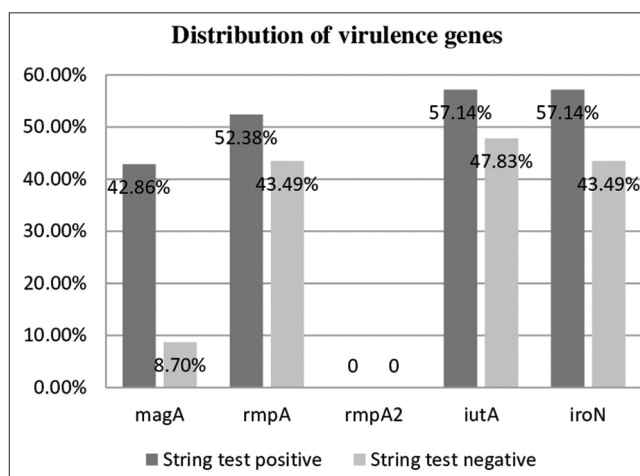
Standards Institute; FDA: Food and Drug Administration;

EUCAST: European Committee on Antimicrobial Susceptibility

Testing

20% in the current study. A study in India reported that 56.9% of samples yielded growth of bacteria.<sup>[15]</sup> The high prevalence rate of *Klebsiella* among clinical patients has become a global concern in recent years since 24% and 19.72% prevalence rates were reported from northeastern and southeastern part of Bangladesh, respectively.<sup>[16,17]</sup> These findings are in accordance with the present study.

In the present study, *K. pneumoniae* was found to be highly resistant to second-, third-, and fourth-generation cephalosporins, aztreonam, and ciprofloxacin. Previous studies in Bangladesh also reported very high resistance rates among isolated *K. pneumoniae* toward cefuroxime, ceftriaxone, ceftazidime, cefepime, and ciprofloxacin.<sup>[18,19]</sup> The resistance pattern of isolated



**Figure 2: The presence of all the virulence genes detected except *rmpA*, was higher in string positive than in string negative strains. However, the distribution of *magA* gene was significantly higher (P = 0.003) among the string positive strains of *K. pneumoniae*. *K. pneumoniae*: *Klebsiella pneumoniae***

*K. pneumoniae* to imipenem (20.45%), tigecycline (20.45%), colistin (31.82%), and fosfomycin (13.67%) in the current study does not correspond to the findings of previous studies in Bangladesh. From 2016 to 2018, the highest resistance developing trend was observed in *K. pneumoniae* toward colistin.<sup>[20]</sup> One systematic review in Bangladesh reported 18.8% median resistance to colistin which ranged from 0% to 21.4%.<sup>[19]</sup> It may be due to the increased use of these drugs in clinical settings coupled with the horizontal transfer of genetic elements including resistance genes. Colistin is also being used unchecked in food, agriculture, poultry, and animal husbandry. One recent study in the US reported that repeated exposure of bacteria to sublethal doses of chlorhexidine which is used invariably in clinical settings might result in cross-resistance with cationic

**Table 5: Distribution of multidrug resistant, extensive-drug resistant, pan-drug resistant, and extended-spectrum beta-lactamases producing *Klebsiella pneumoniae* isolated from different samples (n=44)**

Samples	MDR, n (%)	XDR, n (%)	PDR, n (%)	ESBL, n (%)
Wound swab	6 (13.66)	7 (15.90)	0	2 (33.33)
Urine	3 (6.81)	2 (4.54)	0	1 (16.67)
Sputum	1 (2.26)	0	0	2 (33.33)
ETA	4 (9.09)	1 (2.27)	1 (2.27)	0 (0.00)
Pus	0	0	0	1 (16.67)
Blood	1 (2.27)	0	0	0
Total	15 (34.09)	10 (22.73)	1 (2.27)	6 (13.63)

ETA: Endotracheal aspirates; MDR: Multidrug resistant; XDR: Extensive-drug resistant; PDR: Pandrug resistant; ESBL: Extended-spectrum beta-lactamases

**Table 6: Distribution of virulence genes among isolated *Klebsiella pneumoniae* (N=44)**

Genes	Total, n (%)
<i>magA</i>	11 (25.00)
<i>rmpA</i>	21 (47.73)
<i>rmpA2</i>	0
<i>iutA</i>	23 (52.27)
<i>iroN</i>	21 (47.73)

N=Total number of isolated *K. pneumoniae*; n=Number of different samples positive for different virulence genes. *magA*: Mucoviscosity associated gene A; *K. pneumoniae*: *Klebsiella pneumoniae*

antimicrobials including colistin.<sup>[21]</sup> Furthermore, colistin is often prescribed without knowing the culture and sensitivity report. Most of the microbiology laboratories in Bangladesh still use the disc diffusion method to determine colistin susceptibility patterns whereas detection of MIC is the most accurate method according to the CLSI guidelines.<sup>[9]</sup>

The antibiogram of *Klebsiella* isolates studied in India showed higher resistance against cephalosporins and fluoroquinolones, whereas the highest sensitivity was observed toward imipenem.<sup>[22]</sup> Higher coresistance to cephalosporins as well as quinolones could be because the plasmids that contain the ESBL gene also contain resistance genes for quinolones.<sup>[23]</sup> and availability of these over-the-counter drugs enable people to consume without prescription in incomplete and inappropriate dose regimen. Overall, polymyxin resistance rates in *K. pneumoniae* vary among different regions, but it seems that the prevalence is increasing worldwide.<sup>[24]</sup> Similarly, increased tigecycline resistance has been also observed in *K. pneumoniae* comparing previous studies in Dhaka Medical College Hospital.<sup>[25]</sup>

ESBL-producing Gram-negative bacteria had decreased over time in Bangladesh. Several studies have been conducted and reported the rate of ESBL-producing bacteria as 30.01% in 2008,<sup>[26]</sup> 25% in 2012,<sup>[27]</sup> and 16.01% in 2014.<sup>[28]</sup> In the

present study, 13.64% *K. pneumoniae* were ESBL producers. Changes in the antibiotic prescribing trends such as reduced use of penicillin and cephalosporine along with increased use of imipenem which led to increasing imipenem resistance might be the cause of the low rate of ESBL producers among isolated *K. pneumoniae* in this study. Studies in India have shown that detection rates of ESBL producing *K. pneumoniae* vary from 9.6% to 81.1%.<sup>[29]</sup> It could be due to a varied degree of exposure of this organism to beta-lactam antimicrobials or because of varied transferability of plasmids in the nature or due to the differences in the methodology and interpretation employed.<sup>[29]</sup> The higher rate of ESBL producers in places might be because carbapenem susceptibility pattern or phenotypic metallo-β-lactamase production is not ruled out during screening for ESBL production. However, by definition, ESBL producers must remain sensitive to carbapenems. However, Rao et al.(2014) did not disclose these facts.<sup>[29]</sup>

In this study, 47.73% of the isolated *K. pneumoniae* was string test positive. A study in India reported 31.3% string test positive strains.<sup>[30]</sup> In Egypt, another study reported 40.71% string test positive *K. pneumoniae*.<sup>[31]</sup> The dissimilarity may be due to time, source of sample collection, and geographical variation. Although hypermucoviscosity is associated with virulence, nonhypermucoviscous or string test negative *K. pneumoniae* isolates may also possess the virulence genes and show higher AMR.<sup>[32]</sup> From this, it can be presumed that there are other factors such as K1 and K2 capsular serotypes; ST23 and CC23 sequence types; virulence plasmid pLVPK and KPHP1208 pathogenicity island or other virulence genes that might be responsible for higher resistance rates.<sup>[6]</sup> In the present study, virulence genes *magA* and *rmpA* were detected in 42.86% and 52.38% of isolated string test positive strains, respectively. This finding is in agreement with the study findings in China and Egypt that reported the presence of these genes in 44.4% and 56.52% of the isolated *K. pneumoniae*, respectively.<sup>[31,33]</sup> There are no data available regarding the prevalence of virulence genes and string test positivity in *K. pneumoniae* in Bangladesh or nearby Asian countries.

In the present study, 34.09%, 22.73%, and 2.27% of *K. pneumoniae* isolated from different clinical specimens were found MDR, XDR, and PDR, respectively. These findings coincide with a study in India which reported 30% MDR and 27.8% XDR *K. pneumoniae* isolated from these samples including wound swab, pus, blood, endotracheal aspirates, urine, and sputum.<sup>[34]</sup> A study in Pakistan reported a much higher prevalence of MDR (74.41%), XDR (20.93%), and PDR (4.65%) *K. pneumoniae* which might be because the bacteria were isolated from burn wounds and urine only.<sup>[35]</sup>

## Conclusion

Increasing resistance to last-resort antibiotics such as imipenem, colistin, tigecycline, and fosfomycin with the

high frequency of MDR, XDR, and PDR *K. pneumoniae* observed in this study is alarming. Despite significantly higher frequency of ESBL producers among string test positive strains of isolated *K. pneumoniae* in the present study, no significant association was found regarding MDR and hypervirulence among the two phenotypes. Further studies are required to establish the significance of colistin use in treating patients infected with hypermucoid phenotypes of *K. pneumoniae*. Moreover, routine surveillance of other factors responsible for hypervirulence as well as its association with AMR pattern is needed which may play an important role in public health policymaking.

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### Ethical clearance

Ethical permission for this study was obtained from the Institutional Review board of the corresponding institution, Dhaka Medical College.

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### Conflicts of interest

There are no conflicts of interest.

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